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Simple Method for Repurification of Endotoxins for Biological Use▽

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A method for obtaining highly purified endotoxin (lipopolysaccharide [LPS]) in a few hours by repurification of commercial or laboratory preparations was devised. It avoids the use of phenol, which is not suitable for phenol-soluble lipopolysaccharides nor for some industrial purposes. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry analysis confirmed the integrity of the purified LPSs. The purified products did not activate Toll-like receptor 2 (TLR2), nuclear oligomerization domain 1 (NOD1), or NOD2 but did activate TLR4. Applied to different lipopolysaccharides, the method also improved their mass spectra, thus facilitating their structural analysis.

Lipopolysaccharides (LPSs) are the major components of the external membrane of gram-negative bacteria; they are known as endotoxins and cause several pathophysiological symptoms, such as fever, diarrhea, blood pressure drop, septic shock, and death (11, 19).

LPSs, when isolated from wild-type bacteria, are generally composed of three regions: the hydrophobic lipid A, the oligosaccharide core, and the polysaccharide O-chains. Lipid A, anchored in the external membrane, is responsible for most of the toxic as well as beneficial activities of bacterial endotoxins (29). Its structure is the least variable part of the molecule among the different species of a genus. The core oligosaccharide consists of the proximal "inner core" linked to lipid A and the distal "outer core" carrying the O-chain. The latter, a long polysaccharide, is made of repeating mono- or oligosaccharide units which are highly immunogenic and variable from strain to strain. Because the O-chain structure, when present, gives a smooth aspect to the bacterial colony, its LPS is called S-type LPS. The so-called rough-type bacteria produce LPSs (R-type LPSs) without O-antigens. Lipooligosaccharides are LPSs that have an outer core made of one or more oligosaccharide branches and no O-chain. Some examples are those of *Bordetella pertussis* (9), *Neisseria meningitidis* (10), and *Haemophilus influenzae* (10).

Endotoxins can be isolated from gram-negative bacteria by various methods, the most efficient and commonly used one being the hot phenol-water method (32), which was later modified by certain investigators (2, 14, 21, 22, 33). Usually with this procedure proteins are soluble in the phenol phase and LPSs are soluble in the water phase. However, exceptions exist, and some LPSs are recovered from the phenol phase, for example, the LPSs of *Yersinia enterocolitica* O:9 (4), *Brucella abortus* (5), *Acinetobacter baumannii* (17), and *Leptospira interrogans* (28). They are smooth-type LPSs, and their solubilities can be explained either by the hydrophobicity of the O-chain structure or by conformational reasons, as suggested in the case of *Acinetobacter* LPS (17). A method of extraction specific for rough-type endotoxins using phenol, chloroform, and petroleum ether is also very convenient (13). Among others, a method using precipitation with cold ethanol has also been described (12), but it was suspected by those same authors of discriminating against low-molecular-weight species (11). All the described extraction procedures give endotoxin preparations more or less contaminated with other bacterial components and require additional purification steps to make the preparations usable for biological purposes. These components are phospholipids, proteins, nucleic acids, capsular polysaccharides, peptidoglycan fragments, and lipoproteins. The latter were shown to be responsible for biological activities previously attributed to endotoxins (6, 25). Major improvements in the purification procedures have been made by Manthey and Vogel (26) and Hirschfeld et al. (18). They led to clarification of the endotoxin signaling pathways and showed that Toll-like receptor 2 (TLR2) does not play a role in signaling for most LPS structures, although some exceptions have been reported (15, 16, 28).

However, our interest in LPS preparations isolated from the phenol layer of the phenol-water extraction (27, 28) initiated our search for new conditions. These phenol-soluble LPS preparations could be partly coextracted with all the phenol-soluble contaminants and not easily recovered in their native state by the procedures described. Here we present a quick repurification procedure which can be performed on most rough- and smooth-type preparations, including phenol-soluble LPSs. We were also interested in a phenol-free repurification procedure for use on samples extracted without the use of phenol for some biological purposes.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used for water-soluble LPS preparations were *Escherichia coli* smooth-type 0119 strains NRCC 4182 and 4186 (NRC, Ottawa, Canada) and *Bordetella pertussis* (strain Bp1414) from the Institut

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Mérieux. *Yersinia enterocolitica* strain O:9 from NRC, Ottawa, Canada, was used for phenol-soluble LPS preparation.

LPS extraction and major contaminants removal. LPS preparations were extracted according to the phenol-water procedure described by Westphal and Jann (33). When certain hydrophobic LPSs are extracted by the phenol-water method some, or all, of them may be found in the phenol layer (4). Prudence therefore dictates that both phases be checked for LPS content. LPS preparations are often contaminated with phospholipids, nucleic acids, and lipoproteins. Therefore, RNase, DNase, and then proteinase K (all from Sigma) treatments were performed. Enzymes (1 μg for 1 mg of LPS) were used at the concentration of 5 μg/ml at 37°C for 6 h. The degree of contamination was estimated by UV absorbance at the appropriate wavelengths, and LPS was collected after ultracentrifugation (4°C, 300,000 × *g* for 45 min) and lyophilization. Phospholipid contaminants were removed by extraction from the LPS samples suspended in a chloroform-methanol mixture (1:2, vol/vol) to a concentration of 10 mg/ml. The degree of contamination with phospholipids was controlled by thin-layer chromatography (TLC), as described below. Each procedure was repeated until TLC and spectra of UV absorbance showed no detectable contaminants (8).

Procedures used for removal of TLR2-activating contaminants. (i) TEA-DOC. The triethylamine-deoxycholate sodium (TEA-DOC) phenol-water procedure of repurification was performed as described in reference 18 using TEA as a dissociation agent for comparison.

Three new procedures were tested and compared in this work, detergent, heat, and acid, in order to dissociate LPS-contaminant aggregates. They were used to define the best repurification method.

(ii) Heat-detergent-promoted repurification procedure. LPS preparations (10 mg) were dissolved in aqueous 1% sodium dodecyl sulfate (SDS) solution (0.5 ml) and heated for 5 min at 100°C. After cooling, 6 ml of chloroform and 4 ml of methanol were added. The suspension was sonicated in an ultrasonic bath for 2 min and then centrifuged at 2,000 × *g* for 10 min. The supernatant and the pellet were collected separately. The solvents were evaporated from the supernatant under vacuum, and the recovered material was kept for subsequent tests. The pellet was dried under a stream of nitrogen, and the extraction was repeated twice, replacing SDS solution with water. The dried residue was taken up in water to 5 to 10 mg/ml and ultracentrifuged at 300,000 × *g* for 45 min at 4°C. The lyophilized pellet corresponding to the LPS repurified by the heat-detergent-promoted method was recovered and tested.

(iii) Heat-promoted repurification procedure. LPS preparations were suspended in water at a concentration of 20 mg/ml, adjusted to neutral pH when necessary, and heated at 100°C for 10 min. A mixture of chloroform and methanol was added at room temperature to the cooled solution to attain a final ratio of chloroform, methanol, and water of 3:2:0.25 (vol/vol/vol) and 1 mg LPS/ml. The suspension was treated in an ultrasonic bath for 2 min and then centrifuged at 2,000 × *g* for 10 min. The supernatant was collected and dried under vacuum. The pellet was dried under a stream of nitrogen and reextracted twice more with the same mixture of solvents at room temperature. The dried residue was weighed, taken up in water (5 to 10 mg/ml), and ultracentrifuged at 300,000 × *g* for 45 min at 4°C. The lyophilized pellet corresponding to the LPS repurified by the heat-promoted method was recovered and tested.

(iv) Acid-solvent-promoted repurification procedure (selected procedure). LPS preparations (10 mg) were suspended at room temperature in 1 M hydrochloric acid at a concentration of 20 mg/ml. The suspension was sonicated in an ultrasonic bath for 2 min, and then 6 ml of chloroform and 4 ml of methanol were added in order to obtain a final ratio of the volumes of chloroform, methanol, and 1 M hydrochloric acid of 3:2:0.25 (vol/vol/vol). The suspension was sonicated in an ultrasonic bath for 2 min and then centrifuged at 2,000 × *g* for 10 min. The supernatant and the pellet were separated. The supernatant was evaporated under vacuum. The pellet was dried under a stream of nitrogen, and the extraction was repeated twice, replacing acid with water. When necessary, depending on LPS compositions, two acid extractions were followed by two water extractions as described above (see Discussion, below). After evaporation of the solvent with a stream of nitrogen, the residue was suspended in water (5 to 10 mg/ml) and ultracentrifuged at 300,000 × *g* for 45 min at 4°C. The lyophilized pellet corresponding to the LPS repurified by the acidified solvent-promoted method was recovered and tested.

Detergent-promoted hydrolysis (7). Briefly, the LPSs were cleaved by hydrolysis in 20 mM Na acetate-acetic acid buffer (pH 4.5)–1% SDS at 100°C for 1 h at a concentration of 5 mg/ml. After lyophilization, SDS was extracted once with ethanol–water–1 M HCl (5:1:0.05, vol/vol/vol) and twice with ethanol-water (5:1, vol/vol). The dried residue was suspended in water and centrifuged at 300,000 × *g* for 45 min at 4°C. The lipid A was extracted from the pellet by a mixture of chloroform, methanol, and water (3:2:0.25, vol/vol/vol).

Methods used for testing LPS integrity after the repurification procedure. (i) SDS-polyacrylamide gel electrophoresis of LPS. Gels were loaded with 0.2 to 0.5 μg of R-type LPSs and with 1 to 2 μg of S-type LPSs. Both starting and repurified preparations were electrophoresed as previously described (24) and then stained with silver nitrate (31).

(ii) Thin-layer chromatography. Chromatography was performed on aluminum-backed silica TLC plates (Merck). Ten to 50 μg of LPS or lipid A sample was deposited. A mixture of isobutyric acid–1 M ammonium hydroxide (5:3 [vol/vol]) was used for LPS migration analysis (8). The solvent used for lipid A migration was a mixture of chloroform, methanol, water, and TEA (3:1.5:0.25:0.1 [vol/vol/vol/vol]) as described previously (7). Compounds were visualized by charring (145°C after spraying with 10% sulfuric acid in ethanol).

(iii) MALDI mass spectrometry. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was done on a Voyager-DE STR time-of-flight mass spectrometer (Applied Biosystem). Negative ions were analyzed in the linear mode with delayed extraction. Dihydroxybenzoic acid (Sigma Chemical Co., St. Louis, Mo.) was used as a matrix. A few microliters of a 1-mg/ml water suspension of the LPS or of a 1-mg/ml lipid A solution in chloroform-methanol-water (3:1.5:0.25, vol/vol/vol) was desalted with a few grains of Dowex 50W-X8 (H^+). A 0.5-μl aliquot of the sample was deposited on the target and covered with the same volume of the matrix solution (10 mg/ml of dihydroxybenzoic acid in 0.1 M citric acid solution) (30). The ion acceleration voltage was −20 kV. Extraction delay time was adjusted within 100 to 300 ns to obtain the best spectra. *B. pertussis* or *E. coli* lipid A was used as an external standard for mass calibration.

Biological tests. (i) Reporter assay for NF-κB activation. Human embryonic kidney HEK293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (PAA, Pasching, Austria). For TLR4-dependent NF-κB activation, we used HEK293 cells stably transfected with hTLR4/MD2-CD14 (Invivogen, San Diego, CA). HEK293 cells were seeded into 24-well plates at a density of 10^5 cells/ml, and transfection with various expression plasmids was carried out as described previously (reference 15 and supporting online material). Briefly, HEK293 cells were transfected overnight using 1% FuGENE 6 transfection reagent (Roche Diagnostics, Germany) with 75 ng of the reporter plasmid pNF-κB-Luc (Stratagene, La Jolla, CA) alone or with one of the following vectors: 5 ng nuclear oligomerization domain 1 (NOD1) or NOD2, 20 ng TLR2, and/or TLR1 or TLR6 expression plasmids (Invivogen, San Diego, CA). Total plasmid concentration was 250 ng and was balanced by addition of the pcDNA3.1 vector.

(ii) NOD1 and NOD2 transfection experiments. For NOD1 and NOD2 transfection experiments, the various LPS preparations (1 μg/ml) or muropeptides (diaminopimelic acid-containing muramyl tripeptide [M-triDAP] for NOD1 and muramyl dipeptide [MDP] for NOD2, both at 100 nM) were added to the cell culture medium at the time of transfection, and the synergistic NF-κB-dependent luciferase activation was examined following 16 h of coincubation. M-triDAP was from D. Mengin-Lecreulx (IBBMC, Orsay, France), and MDP was from Sigma (St. Louis, MO). For TLR2- and TLR4-dependent NF-κB activation, the cells were transfected for 16 h and then stimulated for 6 h with the various LPSs at 1 μg/ml and dipalmitoyl *S*-glycerol Cys Ser 4 Lys (Pam2CysSK4) and Pam3CysSK4 at 100 ng/ml (EMC microcollection, Tübingen, Germany) before a luciferase assay. Each test was performed in triplicate.

(iii) Mouse peritoneal macrophage isolation and stimulation. C57BL/6, $TLR2^{-/-}$, and $TLR4^{-/-}$ male mice were used between 8 and 12 weeks of age. The knockout mice were in the C57BL/6 genetic background. Animal care and experimentation were conducted in accord with the Pasteur Institute Animal Care and Use Committee guidelines. Mice were intraperitoneally injected with 2 ml of thioglycolate (Bio-Rad, Marnes-la-Coquette, France). Four days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with ice-cold RPMI 1640 (Glutamax; Cambrex, Rockland, ME). Cells were counted and plated at 0.5 × 10^6/ml for 2 h and washed with RPMI to remove nonadherent cells. Adherent cells were used as peritoneal macrophages, cultured in RPMI supplemented with 1% fetal calf serum, and stimulated with various concentrations of LPS or Pam3CysSK4. After 20 h, supernatants were collected and stored at −20°C. Interleukin-6 (IL-6) concentration in culture supernatants was determined by enzyme-linked immunosorbent assay (DuoSet; R&D Systems, Minneapolis, MN) as specified by the manufacturer.

RESULTS AND DISCUSSION

Preparations to be used for biological assays require a high degree of purity. Rough-type LPS preparations were routinely

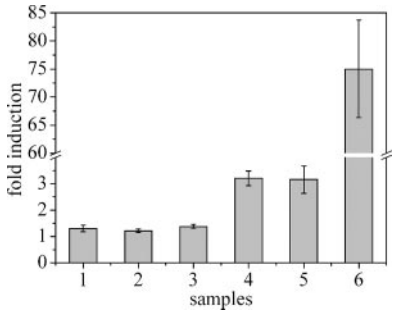

FIG. 1. Comparison of TLR2 activities of the *E. coli* 0119 (strain 4186) LPS preparations repurified by different methods: (1) the method of Hirshfeld et al. (18), reference method; (2) the acid-promoted method; (3) the heat-promoted method; (4) the detergent-promoted method; (5) untreated; (6) Pam3CysSK4. Briefly, HEK293 cells were transfected overnight with a human TLR2 expression vector and an NF-κB-dependent luciferase reporter gene. The cells were then stimulated for 6 h with the various LPS preparations (1 μg/ml) or Pam3CysSK4 (100 ng/ml), and the luciferase activity in cellular lysates was measured. The results are the means ± standard deviations of one experiment representative of three and are expressed as fold induction compared to unstimulated cells.

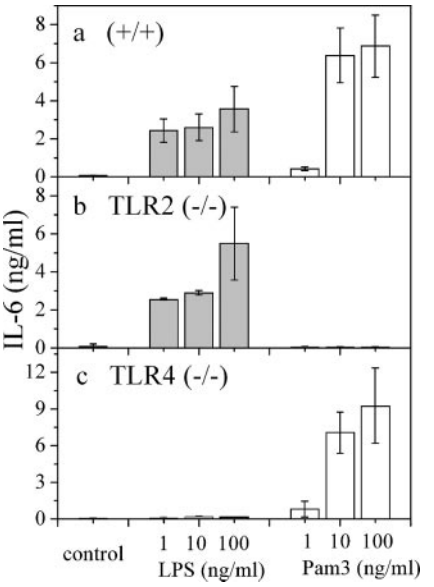

FIG. 2. Assessment of LPS purity using mouse peritoneal macrophages. Thioglycolate-elicited macrophages from C57BL/6 (+/+) (a), $TLR2^{-/-}$ (b), or $TLR4^{-/-}$ (c) mice were stimulated for 20 h with various concentrations of LPS from *E. coli* 0119 repurified by the new acid-promoted method or by Pam3CysSK4. The concentration of IL-6 in culture supernatants was determined by enzyme-linked immunosorbent assay. The results are expressed as means ± standard errors of the means of three independent experiments.

tested in this laboratory on TLC to check their degree of heterogeneity and purity (8). Nucleic acid and/or protein contaminants are detected on the baseline and can be removed by enzymatic treatments as described in Materials and Methods. Polysaccharide contaminants, usually also remaining on the baseline, can be removed by repeated ultracentrifugation in water with or without the addition of NaCl (1 M). Phospholipids and free fatty acids migrating to the upper part of the TLC can be extracted with the relevant mixture of solvents.

These procedures are usually efficient for the elimination of the major contaminants, and the resulting LPSs are suitable for structural analyses. An additional repurification procedure was described for biological purposes (18, 26) and found efficient and convenient for most samples except for the phenol-soluble LPS (27, 28).

Repurification procedures and biological assays. The efficiency of a given purification procedure seemed to depend on the physico-chemical interactions occurring between LPS and different membrane components.

A preliminary experiment showed that after hydrolysis of an LPS preparation to separate the lipid A from the polysaccharide, the TLR2 signaling component is coextracted with the lipid A fractions. This suggested that the contaminants were strongly associated with the lipid A moiety in the LPS. In order to remove such contaminants from the LPS preparations, we tried three different dissociation agents, heat, detergent (SDS), and mineral acid, before the relevant solvent extraction process.

The three procedures were applied to the smooth-type *E. coli* 0119 LPS (1) as described in Materials and Methods, and the repurified samples were compared with that obtained using the method of Hirschfeld et al. (18). The efficiency of the repurification procedures was estimated using HEK293 cells overexpressing TLR2 and transfected with an NF-κB-dependent luciferase reporter gene. (Fig. 1). Our data showed a decrease in TLR2 activation obtained with the repurified products compared to that of the starting sample. No change was observed with the SDS-heat procedure; the two others gave nearly similar results, but the acid treatment gave the lowest TLR2 activation and the best-purified LPSs (Fig. 1).

In order to confirm that contaminants were not overlooked with the HEK293 transfected cells, we used mouse peritoneal macrophages, which are very sensitive sensors of bacterial products. Thioglycolate-elicited macrophages from C57BL/6 (+/+) $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were stimulated by various concentrations of both purified LPS and Pam3CysSK4. The results are presented in Fig. 2 and clearly demonstrate that the purified LPS induced IL-6 via TLR4 and not via TLR2, whereas Pam3CysSK4 induced IL-6 via TLR2.

An additional experiment, illustrated in Fig. 3, was performed to show that the transfected cells were efficiently activated by Pam2CysSK4, a diacylated lipopeptide known to be sensed by the TLR2/6 heterodimer. As shown in Fig. 3a, the purified LPS did not activate the cells via TLR2, while Pam3CysSK4 as well as Pam2CysSK4 gave good results, attesting to the efficiency of the present model for lipoprotein detection. As di- and triacylated lipopeptides are detected by TLR2/TLR6 and TLR2/TLR1 heterodimers, respectively, we evaluated the impact of TLR1 or TLR6 cotransfection on the sensitivity of our detection system. Coexpression of TLR1 or TLR6 with TLR2 only moderately increased the cellular response to lipopeptides. This may be explained by the fact that HEK293 cells endogenously express TLR1 and TLR6 and respond to lipopeptides when only transfected with TLR2 (20, 23). It has been shown that with some lipopeptides TLR1 and TLR2 expression is sufficient for a cellular response (3). Finally, the activity of our repurified LPS was assessed using HEK293 cells stably expressing TLR4-CD14-MD2 and trans-

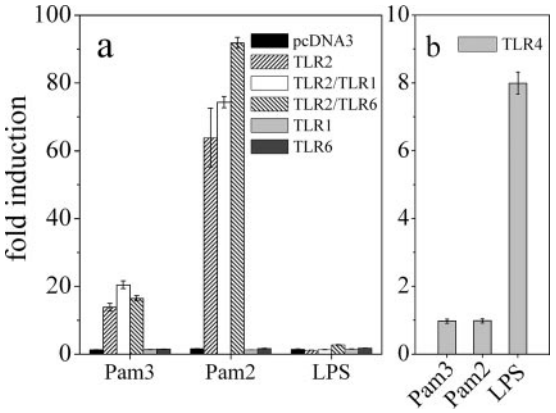

FIG. 3. Detection of di- as well as triacylated lipopeptides by TLR2-transfected HEK293 cells. (a) HEK293 cells were transfected overnight with a human TLR2 expression vector alone or in the presence of human TLR1 or TLR6 and of an NF-κB-dependent luciferase reporter gene. The cells were then stimulated for 6 h with an LPS preparation (*E. coli* 4182 repurified LPS) at 1 μg/ml, Pam2CysSK4 (100 ng/ml), or Pam3CysSK4 (100 ng/ml), and the luciferase activity in cellular lysates was measured. The results are the means ± standard deviations of one experiment representative of three. (b) Activity of the purified LPS was checked using HEK293 cells stably expressing human TLR4, CD14, and MD-2 and transfected overnight with an NF-κB-dependent luciferase reporter gene. The conditions of stimulation and luciferase measurement were the same as for panel a.

fected with a luciferase reporter gene under the control of NF-κB. As shown in Fig. 3b, our purified LPS activated NF-κB in these cells, whereas no activation was observed with the TLR2 agonists Pam3CysSK4 and Pam2CysSK4.

The acidic method was then selected and used in the following experiments. It was applied to other LPS preparations, including the lipooligosaccharide from *B. pertussis* and two

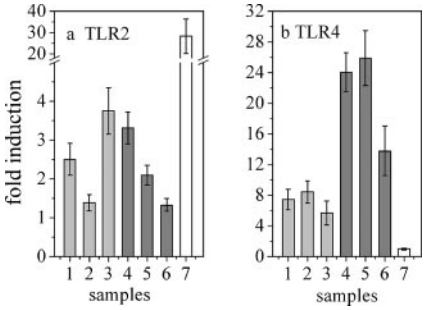

FIG. 4. Comparison of the TLR2 and TLR4 activities of samples before and after repurification by the new acid-promoted method. Columns 1 to 3: *B. pertussis* LPS starting material purified as previously described in reference 9 (1), repurified (2), or extracted contaminants (3). Columns 4 to 6, *Y. enterocolitica* O:9 LPS starting material purified as previously described in reference 4 (4), repurified (5), extracted contaminants (6). Column 7, Pam3CysSK4. (a) HEK293 cells were transfected overnight with a human TLR2 expression vector and an NF-κB-dependent luciferase reporter gene. (b) HEK293 cells stably expressing human TLR4, CD14, and MD-2 were transfected overnight with an NF-κB-dependent luciferase reporter gene. In both cases, after the transfection the cells were stimulated for 6 h with the various LPS preparations (1 μg/ml) or Pam3CysSK4 (100 ng/ml) and the luciferase activities in cellular lysates were measured. The results are the means ± standard deviations of one experiment representative of three and are expressed as fold induction compared to unstimulated cells.

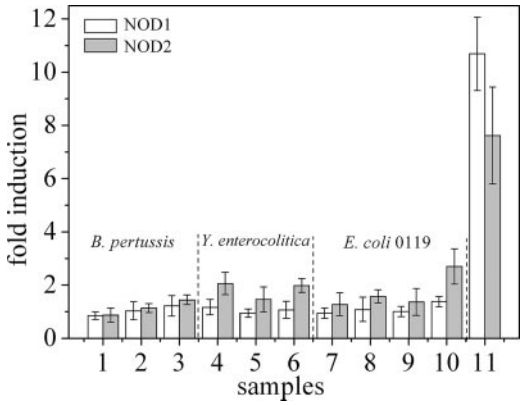

FIG. 5. NOD1 and NOD2 activities of samples before and after repurification. Columns 1 to 3, *B. pertussis* LPS (1 μg/ml) as starting material purified as previously described in reference 9 (1), repurified by the new acid-promoted method (2), or contaminants extracted by the new acid-promoted method (3). Columns 4 to 6, *Y. enterocolitica* O:9 LPS starting material purified as previously described in reference 4 (4), repurified by the acid-promoted method (5), or contaminants extracted by the acid-promoted method (6). Columns 7 to 10, *E. coli* 0119 strain 4182 LPS starting material purified as previously described in reference 1 (7), repurified according to Hirshfeld's method (see reference 18), reference method (8), repurified by the acid-promoted method (9), or contaminants extracted by the acid-promoted method (10). Column 11, M-triDAP at 100 nM for NOD1 and muramyl dipeptide at 100 nM for NOD2. HEK293 cells were transfected overnight with human NOD1 or NOD2 expression vectors and an NF-κB-dependent luciferase reporter gene. The various agonists were added to the culture medium at the time of transfection, and luciferase activation was examined after 16 h. The results are the means ± standard deviations of one experiment representative of three and are expressed as fold induction compared to unstimulated cells.

phenol-water-extracted LPSs, the water-soluble LPS of *E. coli* and the phenol-soluble LPS of *Y. enterocolitica*.

The efficiency of acid for the dissociation between lipid A and other noncovalently associated components was demonstrated previously with the dissociation of lipid A and SDS after the SDS-promoted hydrolysis (7).

The extracted contaminants were also tested and shown to be responsible for TLR2 signaling, as demonstrated in Fig. 4. The treated *B. pertussis* LPS preparation showed that the TLR2 activating power is absent and that the extract has increased TLR2 stimulatory activity. Similar results were obtained with the smooth-type *Y. enterocolitica* LPS, although the extract gave a weaker response because of difficulties in weighing this minor and sticky extracted material.

The contaminants commonly found in commercial preparations of LPS include lipoproteins that signal through TLR2 and peptidoglycan, which can activate cells via NOD1 or NOD2. To evaluate possible contamination of our LPS preparations, we used HEK293 cells transfected with NOD1 or NOD2 and an NF-κB-dependent luciferase gene. As shown in Fig. 5, a very low NF-κB activation (twofold increase) was observed with some of the starting LPS preparations. However, when present, this activity was removed by repurification and found only in the chloroform-methanol-water fraction containing the contaminants. As positive controls, we used M-triDAP for NOD1 and muramyl dipeptide for NOD2. These results attest to the absence of peptidoglycan contamination of the LPS.

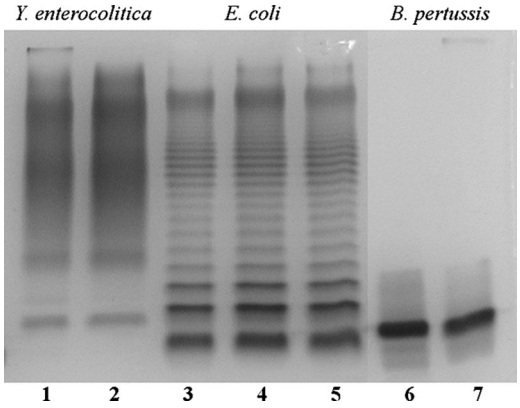

FIG. 6. SDS-polyacrylamide gel electrophoresis of LPS samples before and after repurification. Lanes 1 and 2, *Y. enterocolitica* O:9 (starting material [1] or repurified by the acid-promoted method [2]); lanes 3 to 5, *E. coli* 0119 strain 4182 (starting material [3], repurified by the acid-promoted method [4], or repurified according to the method of Hirshfeld et al. as described in reference 18 [5]); lanes 6 and 7, *B. pertussis* (starting material [6] or repurified by the acid-promoted method [7]).

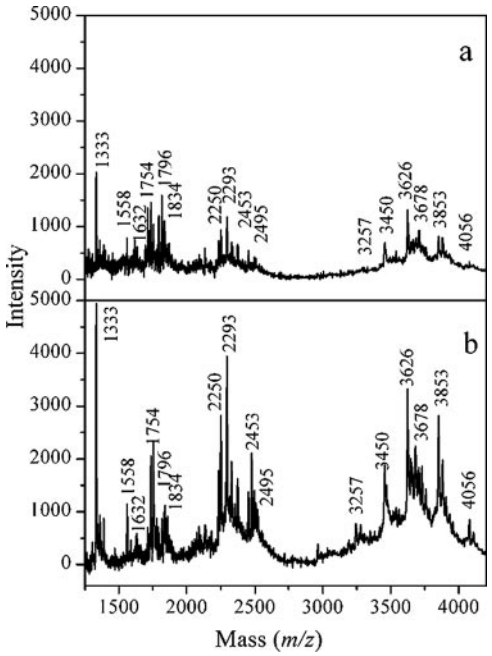

FIG. 7. Comparison of negative ion MALDI-mass spectra of *B. pertussis* LPS samples before and after repurification by the new acid-promoted method: (a) starting LPS; (b) repurified LPS.

The purified LPS preparations lost their TLR2-activating contaminants but conserved and/or increased their capacity to activate TLR4 as shown in Fig. 4. This was interpreted as the first evidence for their conserved integrity. Experiments with HEK293 cells transfected with NOD1 and NOD2 attested to the absence of peptidoglycan contamination (Fig. 5).

Phenol-soluble extracted LPS preparations like those of *Y. enterocolitica* proved to be more difficult to purify than the water-soluble LPS preparations. They had an absolute requirement for the use of two steps of acid solvent extraction. On the other hand, most LPSs could be purified by only one acidic treatment. But as no two LPS preparations are exactly alike, because of varied heterogeneity and a wide diversity of constituents, we propose that two acid extractions be done for security's sake. Nevertheless, different methods should be compared for use with any given preparation, and biological tests should be performed to attest to the appropriate selection. Until now, no method has been described for the repurification of phenol-soluble LPS preparations, and methods using phenol for extracting contaminants cannot be used with these peculiar preparations.

Yield and integrity of repurified LPS preparations. The different LPS preparations were recovered with a yield of 85 to 90%. Considering the various steps of the procedure leading to the removal of contaminants and to minor losses, these yields were found acceptable. In order to be sure that no modification of the starting LPS occurred and that no particular molecular species was selectively removed, the resulting LPSs were also analyzed by various analytical methods: TLC and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed on each extract and after each procedure. LPS and lipid A molecular compositions were checked by mass spectrometry.

SDS-PAGE control. Comparisons of LPS profiles from *E. coli*, *B. pertussis*, and *Y. enterocolitica*, before and after the selected repurification, were performed by SDS-PAGE and silver nitrate staining (31). They showed no modifications of the sample profiles, as seen in Fig. 6.

MALDI-mass spectrometry control. It was observed that the selected repurification process was also beneficial to the analysis of LPSs by mass spectrometry, as shown in Fig. 7 with the example of *B. pertussis* LPS (9). We compared spectra of *Bordetella pertussis* LPS obtained with (Fig. 7b) and without (Fig. 7a) the repurification method. Identical mass spectrometry conditions were used and showed a three to fourfold increase in signal intensity after repurification. We consider this improvement to be due to a better dissociation between molecular species and the removal of contaminants able to increase LPS aggregation and to hamper desorption and ionization. The presence of the same peaks, corresponding to LPS molecular ion species (*m*/*z* 3,257 to 4,056), polysaccharide ions (*m*/*z* 1,632 to 2,495), and lipid A ions (*m*/*z* 1,333 and 1,558) as described in the structural report (9), was observed in the spectra before and after repurification, confirming that no modification of the starting LPS occurred.

Lipid A, known to be responsible for the majority of the biological activities elicited by the whole molecule, was isolated from different LPS preparations before and after the selected repurification and analyzed by MALDI-mass spectrometry. As illustrated with the example of *E. coli* (Fig. 8), no modifications and discriminations occurred for different molecular species, confirming the suitability of the process.

The materials extracted from the LPS preparations were characterized only by their biological activities. They are assumed to be peptidoglycan fragments or lipoproteins (25, 26).

The method described fulfilled the objectives of this work: the resulting LPS was not structurally modified, nor was its

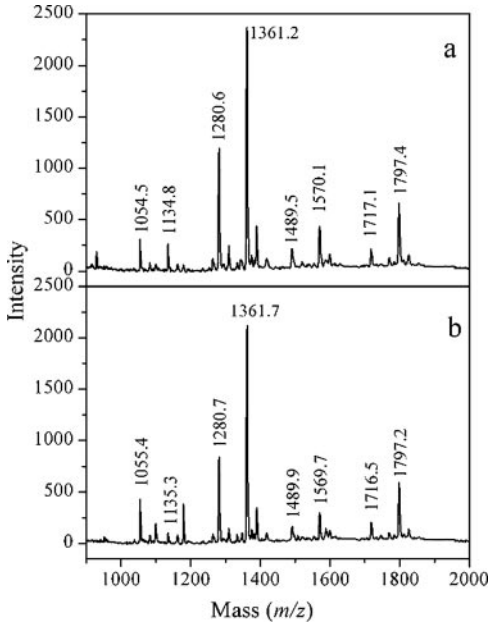

FIG. 8. Comparison of negative ion MALDI-mass spectra of *E. coli* lipid A isolated from enzyme-treated LPS (a) or repurified LPS (b).

heterogeneity profile altered as seen by SDS-PAGE and mass spectrometry.

At least three characteristics were appreciated in the proposed repurification method: it is rapid, taking only a few hours, uses very common solvents, and can be used to purify phenol-soluble as well as water-soluble LPSs. The absence of phenol makes the method attractive for other purposes, such as research on pharmaceutics, vaccines, and biological activities.

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